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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 31/00	A1	(11) International Publication Number: WO 96/14837 (43) International Publication Date: 23 May 1996 (23.05.96)
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(54) Title: GENE THERAPY FOR HYPERCHOLESTEROLEMIA (57) Abstract An adenoviral vector including a nucleic acid sequence encoding apolipoprotein E or a fragment or derivative thereof. Such adenoviral vectors may be administered to a host as part of a gene therapy treatment of hypercholesterolemia.		

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GENE THERAPY FOR HYPERCHOLESTEROLEMIA

This invention relates to hypercholesterolemia and the treatment thereof. More particularly, this invention relates to the treatment of hypercholesterolemia through the administration of adenoviral vectors including DNA encoding apolipoprotein E.

BACKGROUND OF THE INVENTION

Apolipoprotein E (also known as Apo E) is a component of several plasma lipoproteins including chylomicrons, VLDL, and HDL. Receptor-mediated catabolism of lipoprotein particles is mediated through the interaction of Apo E with the LDL receptor or with the LDL receptor-related protein, LRP (Mahley, Science, Vol. 240, pgs. 622-630 (1988); Willnow, et al., Science, Vol. 264, pgs. 1471-1474 (1994)). Injection of exogenous Apo E into normal and hyperlipidemic rabbits resulted in a decrease in plasma cholesterol concentrations. (Yamada, et al., Proc. Nat. Acad. Sci., Vol. 86, pgs. 665-669 (1989); Mahley, J. Clin. Invest., Vol. 83, pgs. 2125-2130 (1989)). These studies demonstrated that elevation of Apo E levels in plasma could apparently increase the clearance of lipoproteins from the circulation. The cholesterol lowering effect due to the intravenous injection of Apo E, however, was transient and lasted about 20 hours. In Apo E transgenic mice, stable overexpression of Apo E resulted in a sustained reduction of plasma cholesterol concentrations and resistance

to dietary elevation of plasma cholesterol concentrations (Shimano, et al., Proc. Nat Acad. Sci., Vol. 89, pgs. 1750-1754 (1992)). Kinetic studies of VLDL, LDL, and chylomicrons in Apo E transgenic mice showed that the overexpression of Apo E enhanced the clearance of these lipoproteins from the circulation (Shimano, et al., J. Clin. Invest., Vol. 90, pgs. 2084-2091 (1992), Shimano, et al., J. Clin. Invest., Vol. 93, pgs. 2215-2223 (1994)). Taken together, these studies support the hypothesis that overexpression of Apo E will reduce plasma cholesterol and/or triglyceride concentrations by increasing the clearance of plasma lipoproteins from the circulation.

Apo E-deficient mice demonstrate further the physiologic importance of Apo E in lipoprotein metabolism. Apo E-deficient mice are severely hypercholesterolemic with average plasma cholesterol concentrations of 400 to 800 mg/dl on a regular chow diet. (Zhang, et al., Science, Vol. 258, pgs. 468-471 (1992); Plump, Cell, Vol. 71, pgs. 343-351 (1992)). These mice also develop atherosclerotic lesions at approximately 11 weeks of age with the appearance of foam cells which progress to a more involved, complex lesion including cholesterol clefts and fibrous caps (Redderick, et al., Arterioscler. Thromb., Vol. 14, pgs. 141-147 (1994)). The severe hypercholesterolemia found in Apo E-deficient mice and the profound effect of deleting the Apo E gene on lipoprotein metabolism demonstrates that Apo E plays a key role in the receptor mediated clearance of plasma lipoproteins through its interaction with either the LDL receptor or the LRP.

It is therefore an object of the present invention to treat hypercholesterolemia in an individual by providing the individual with an expression vehicle including a lipoprotein gene.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described with respect to the drawings, wherein:

Figure 1 is a map of plasmid pCRII;

Figure 2 is a map of plasmid pCRE;

Figure 3 is a schematic of the construction of plasmid pHR;

Figure 4 is a schematic of the construction of an adenoviral vector including an adenoviral ITR, an E1a enhancer sequence, a Rous Sarcoma Virus promoter, and an Adenovirus 5 tripartite leader sequence;

Figure 5 is a schematic of the construction of plasmid pAvS6;

Figure 6 is a plasmid map of plasmid pAvS6;

Figure 7 is a map of plasmid pAvS6E;

Figure 8 is a schematic of the construction of Av1RE;

Figure 9 is a map of plasmid pAvS6-nLacZ;

Figure 10 is a schematic of the construction of Av1LacZ4;

Figure 11 is a Western blot of in vitro human apoE expression from HepG2 cells transduced with Av1LacZ4 or Av1RE;

Figure 12 is a Western blot of in vivo expression of human apoE in apoE-deficient mice treated with Av1LacZ4 or Av1RE;

Figure 13 is a graph of plasma cholesterol concentrations in apoE-deficient mice treated with Av1LacZ4 or Av1RE at 0 and 7 days post-infection;

Figure 14 is a graph of plasma lipoprotein distributions in apoE-deficient mice treated with Av1LacZ4 or Av1RE;

Figure 15 is a Western blot of human apoE distribution among mouse plasma lipoproteins in mice treated with Av1RE; and

Figures 16A and 16B are graphs of total plasma cholesterol and plasma human apoE concentrations, respectively, in mice treated with Av1LacZ4 or Av1RE.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with an aspect of the present invention, there is provided an adenoviral vector including a nucleic acid sequence encoding apolipoprotein E or a fragment or derivative thereof having the biological activity of apolipoprotein E.

Apolipoprotein E results in cholesterol lowering through an increase in the receptor mediated catabolism of apolipoprotein B containing lipoprotein particles.

The term "fragment or derivative thereof" as used herein means that the apolipoprotein E may be a protein which has deletion(s) of amino acid residues within the protein structure, and/or may be truncated at the C-terminal and/or the N-terminal, and/or may be mutated such that one or more amino acid residues normally present in the protein structure are replaced with other amino acid residues. Such fragments and derivatives of apolipoprotein E retain the same biological activity as unmodified apolipoprotein E.

The nucleic acid sequence encoding apolipoprotein E or a fragment or derivative thereof may be the native nucleic acid sequence which encodes apolipoprotein E or a fragment or derivative thereof, or an allelic variant thereof. The term "allelic variant" as used herein means that the allelic variant is an alternative form of the native nucleic acid sequence which may have a substitution, deletion, or addition of one or more nucleotides, which does not alter substantially the function of the encoded apolipoprotein E or fragment or derivative thereof.

In a preferred embodiment, the nucleic acid sequence encoding apolipoprotein E or a fragment or derivative thereof encodes human apolipoprotein E, and in particular, human

apolipoprotein E3. The nucleic acid sequence preferably is a DNA sequence, which may be a cDNA sequence or a genomic DNA sequence. The DNA sequence encodes the full length apolipoprotein E or may encode a fragment or derivative of apolipoprotein E, and the DNA sequence may further include a leader sequence or portion thereof, a secretory signal or portion thereof of the apolipoprotein E gene, and/or may further include a trailer sequence or portion thereof of the apolipoprotein E gene.

Applicants have found unexpectedly that, when animal hosts were infected with adenoviral vectors including a nucleic acid sequence encoding apolipoprotein E, that in such animal hosts, one obtains long term expression of the nucleic acid sequence encoding apolipoprotein E, as well as long term lowering of plasma cholesterol concentrations.

The adenoviral vector which is employed may, in one embodiment, be an adenoviral vector which includes essentially the complete adenoviral genome. (Shenk, et al., Curr. Top. Microbiol. Immunol., (1984); 111(3):1-39). Alternatively, the adenoviral vector may be a modified adenoviral vector in which at least a portion of the adenoviral genome has been deleted.

In one embodiment, the vector comprises an adenoviral 5' ITR; an adenoviral 3' ITR; an adenoviral encapsidation signal; a DNA sequence encoding apolipoprotein E or a fragment or derivative thereof; and a promoter controlling the DNA sequence encoding apolipoprotein E or a fragment or derivative thereof. The vector is free of at least the majority of adenoviral E1 and E3 DNA sequences, but is not free of all of the E2 and E4 DNA sequences, and DNA sequences encoding adenoviral proteins promoted by the adenoviral major late promoter.

In still another embodiment, the gene in the E2a region that encodes the 72 kilodalton binding protein is mutated to produce a temperature sensitive protein that is active at

32°C, the temperature at which viral particles are produced, but is inactive at 37°C, the temperature of the animal or human host. This temperature sensitive mutant is described in Ensinger, et al., J.Virology, 10:328-339 (1972); Van der Vliet, et al., J.Virology, 15:348-354 (1975); and Friefeld, et al., Virology, 124:380-389 (1983); Englehardt, et al., Proc.Nat.Acad.Sci., Vol. 91, pgs. 6196-6200 (June 1994); Yang, et al., Nature Genetics, Vol. 7, pgs. 362-369 (July 1994).

Such a vector, in a preferred embodiment, is constructed first by constructing, according to standard techniques, a shuttle plasmid which contains, beginning at the 5' end, the "critical left end elements," which include an adenoviral 5' ITR, an adenoviral encapsidation signal, and an E1a enhancer sequence; a promoter (which may be an adenoviral promoter or a foreign promoter); a tripartite leader sequence, a multiple cloning site (which may be as herein described); a poly A signal; and a DNA segment which corresponds to a segment of the adenoviral genome. Such DNA segment serves as a substrate for homologous recombination with a modified or mutated adenovirus, and such sequence may encompass, for example, a segment of the adenovirus 5 genome no longer than from base 3329 to base 6246 of the genome. The plasmid may also include a selectable marker and an origin of replication. The origin of replication may be a bacterial origin of replication. Representative examples of such shuttle plasmids include pAvS6, shown in Figure 6. A desired DNA sequence encoding a therapeutic agent may then be inserted into the multiple cloning site to produce a plasmid vector.

This construct then is used to produce an adenoviral vector. Homologous recombination then is effected with a modified or mutated adenovirus in which at least the majority of the E1 and E3 adenoviral DNA sequences have been deleted. Such homologous recombination may be effected through co-

transfection of the plasmid vector and the modified adenovirus into a helper cell line, such as 293 cells, by CaPO_4 precipitation. Upon such homologous recombination, a recombinant adenoviral vector is formed which includes DNA sequences derived from the shuttle plasmid between the Not I site and the homologous recombination fragment, and DNA derived from the E1 and E3 deleted adenovirus between the homologous recombination fragment and the 3' ITR.

In one embodiment, the homologous recombination fragment overlaps with nucleotides 3329 to 6246 of the adenovirus 5 (ATCC VR-5) genome.

Through such homologous recombination, a vector is formed which includes an adenoviral 5' ITR, an adenoviral encapsidation signal; an E1a enhancer sequence; a promoter; a tripartite leader sequence; a DNA sequence encoding apolipoprotein B or a fragment or derivative thereof; a poly A signal; adenoviral DNA free of at least the majority of the E1 and E3 adenoviral DNA sequences; and an adenoviral 3' ITR. This vector may then be transfected into a helper cell line, such as the 293 helper cell line (ATCC No. CRL1573), which will include the E1a and E1b DNA sequences, which are necessary for viral replication, and to generate infectious viral particles. Transfection may take place by electroporation, calcium phosphate precipitation, microinjection, or through proteoliposomes.

The vector hereinabove described may include a multiple cloning site to facilitate the insertion of DNA sequence(s) encoding lipoprotein(s) into the cloning vector. In general, the multiple cloning site includes "rare" restriction enzyme sites; i.e., sites which are found in eukaryotic genes at a frequency of from about one in every 10,000 to about one in every 100,000 base pairs. An appropriate vector is thus formed by cutting the cloning vector by standard techniques at appropriate restriction sites in the multiple cloning

site, and then ligating the DNA sequence encoding a lipoprotein(s) into the cloning vector.

The DNA sequence encoding apolipoprotein E or fragment or derivative thereof is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the Rous Sarcoma Virus (RSV) promoter; inducible promoters, such as the MMTV promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoE promoter; and the ApoAI promoter. It is to be understood, however, the scope of the present invention is not limited to specific foreign genes or promoters.

In one embodiment, the adenovirus may be constructed by using a yeast artificial chromosome (or YAC) containing an adenoviral genome according to the method described in Ketner, et al., PNAS, Vol. 91, pgs. 6186-6190 (1994), in conjunction with the teachings contained herein. In this embodiment, the adenovirus yeast artificial chromosome is produced by homologous recombination *in vivo* between adenoviral DNA and yeast artificial chromosome plasmid vectors carrying segments of the adenoviral left and right genomic termini. A DNA sequence encoding apolipoprotein E or a fragment or derivative thereof then may be cloned into the adenoviral DNA. The modified adenoviral genome then is excised from the adenovirus yeast artificial chromosome in order to be used to generate infectious adenoviral particles as hereinabove described.

The infectious viral particles may then be administered in vivo to a host. The host may be an animal host, including mammalian, non-human primate, and human hosts. The particular target cell or tissue to which the particles are targeted is dependent upon the ligand with which the particle is engineered.

The viral particles are administered in an amount effective to treat hypercholesterolemia in a host, i.e., the administration of the viral particles provides for the lowering of cholesterol levels in the blood of the host. In one embodiment, the viral particles may be administered in an amount of from 1 plaque forming unit to about 10^{14} plaque forming units, preferably from about 10^6 plaque forming units to about 10^{13} plaque forming units.

Hypercholesterolemia often is associated with cardiovascular disease, such as, for example, atherosclerosis, which is the blocking of arteries as a result of cholesterol deposits. Thus, the adenoviral vectors of the present invention are useful in the prevention and/or treatment of cardiovascular disease, wherein such vectors are administered in the amounts hereinabove described.

Preferably, the infectious vector particles are administered systemically, such as, for example, by intravenous administration (such as, for example, portal vein injection or peripheral vein injection), or intramuscular administration.

The viral particles may be administered in combination with a pharmaceutically acceptable carrier suitable for administration to a patient. The carrier may be a liquid carrier (for example, a saline solution), or a solid carrier, such as, for example, microcarrier beads.

In another embodiment, the viral particles may be administered to apolipoprotein-deficient animals (such as, for example, apoE-deficient mice) in order to use such animals as models for studying hypercholesterolemia and the treatment thereof. For example, an adenoviral vector containing the apoE gene may be given to apoE-deficient mice. Subsequent to the administration of the adenoviral vector containing the apoE gene, the mice are evaluated for expression of apolipoprotein E. From the results of such a study, one then may determine how such adenoviral vectors may

be administered to human patients for the treatment of apolipoprotein-E deficiency. Thus, the adenoviral vectors of the invention are useful as scientific reagents in the study of hypercholesterolemia.

In another alternative, the adenoviral vector may be employed to transduce eukaryotic cells in vitro, whereby the eukaryotic cells will produce apolipoprotein E in vitro. Eukaryotic cells which may be transduced include any eukaryotic cell type, and, in particular, include hepatocytes, endothelial cells, and primary cells, such as primary nucleated blood cells. Such apolipoprotein E then may be obtained from the culture of transduced eukaryotic cells, and may be administered to a host for the treatment of hypercholesterolemia.

In another alternative, the eukaryotic cells, which were transduced with the adenoviral vector in vitro, may be administered to a host in an amount effective to treat hypercholesterolemia in a host.

EXAMPLES

The invention now will be described with respect to the following examples; however, the scope of the present invention is not intended to be limited thereby.

Example 1

Construction of Adenoviral Vector Including DNA Encoding Apolipoprotein E

A. Cloning of Apolipoprotein E cDNA

Apolipoprotein E (ApoE) cDNA was constructed using gene overlap extension PCR methods (Horton, et al., Biotechniques, Vol. 8, pgs. 528-535 (1990)), using Pfu DNA polymerase (Stratagene, La Jolla, CA) in the presence of 10% DMSO. The 5' end of the apoE cDNA, nucleotides-39 to 292, was generated using human liver cDNA (Clontech, Palo Alto, CA) as template with the following primers:

P1: 5'-ACTCAGCCCCAGCGGAGGTGAAGGACGTCCTTCCCCAGGAGCCG-3'

P2: 5'-TTCCTCCAGTTCGATTGTAGGCCTTCAACTCCTTCATGGTCTCGTC-3'

The primer P1 was designed to start at the major transcription initiation site in Exon 1 (Paik, et al., Proc. Nat. Acad. Sci., Vol. 82, pgs. 3445-3449 (1985)). The PCR reaction was carried out using the following conditions: 95°C for 10 min., followed by 30 cycles of 95°C for 30 seconds, 60°C for 1 minute, 72°C for 2 minutes, and finally a 72°C extension for 10 minutes. The 3' end of the apoE cDNA was amplified from a cloned apoE fragment, EB4 (Wallis, et al., EMBO J., Vol. 2, pgs. 2369-2373 (1983)) obtained from Dr. Steven Humphries using the primers P3 (5'-GCCTACAAATCGGAACTGGAGGAA-3') and P4 (5'-AGGCTTCGGCGTTCACTGATTGT-3') to produce a 696 bp fragment. The 5' and 3' PCR generated apoE fragments were gel purified. The PCR reaction was set up using equal volumes of the melted fragments and the end primers P1 and P4. The expected full length apoE cDNA, 1,025 bp, was amplified and was ligated directly into the pCRII vector (Figure 1) (Invitrogen, San Diego, CA) to form pCRE (Figure 2). The apoE cDNA sequence includes a leader sequence of 60 bp 5' to the ATG start codon, a secretion signal peptide coding region, the apoE3 coding region, and a trailer sequence of 93 bp 3' to the stop codon. This sequence corresponds to bp-2 to bp 1,026 of the apoE3 sequence of Genbank accession #K00396. Several clones were screened by restriction enzyme analysis and then were sequenced. A clone having a perfect match with the expected sequence (Genbank accession #K00396) was selected.

B. Construction of pAvS6.

The adenoviral construction shuttle plasmid pAvS6 was constructed in several steps using standard cloning techniques including polymerase chain reaction based cloning techniques. First, the 2913 bp BglIII, HindIII fragment was removed from Ad-d1327 and inserted as a blunt fragment into the XhoI site of pBluescript II KS (Stratagene, La Jolla, CA) (Figure 3). Ad-d1327 (Thimmappaya, et al., Cell, Vol. 31,

pgs. 543-551 (1983), incorporated herein by reference) is identical to Adenovirus 5 except that an XbaI fragment including bases 28591 to 30474 (or map units 78.5 to 84.7) of the Adenovirus 5 genome, and which is located in the E3 region, has been deleted. The complete Adenovirus 5 genome is registered as Genbank accession #M73260, incorporated herein by reference, and the virus is available from the American Type Culture Collection, Rockville, Maryland, U.S.A. under accession number VR-5.

Ad-dl327 was constructed by routine methods from Adenovirus 5 (Ad5). The method is outlined briefly as follows and previously described by Jones and Shenk, Cell 13:181-188, (1978). Ad5 DNA is isolated by proteolytic digestion of the virion and partially cleaved with XbaI restriction endonuclease. The XbaI fragments are then reassembled by ligation as a mixture of fragments. This results in some ligated genomes with a sequence similar to Ad5, except excluding sequences 28591 bp to 30474 bp. This DNA is then transfected into suitable cells (e.g. KB cells, HeLa cells, 293 cells) and overlaid with soft agar to allow plaque formation. Individual plaques are then isolated, amplified, and screened for the absence of the 1878 bp E3 region XbaI fragment.

The orientation of this fragment was such that the BglIII site was nearest the T7 RNA polymerase site of pKSII and the HindIII site was nearest the T3 RNA polymerase site of pBluescript II KS. This plasmid was designated pHR. (Figure 3).

Second, the ITR, encapsidation signal, Rous Sarcoma Virus promoter, the adenoviral tripartite leader (TPL) sequence and linking sequences were assembled as a block using PCR amplification (Figure 4). The ITR and encapsidation signal (sequences 1-392 of Ad-dl327 [identical to sequences from Ad5, Genbank accession #M73260], incorporated herein by reference) were amplified

(amplification 1) together from Ad-d1327 using primers containing NotI or AscI restriction sites. The Rous Sarcoma Virus LTR promoter was amplified (amplification 2) from the plasmid pRC/RSV (sequences 209 to 605; Invitrogen, San Diego, CA) using primers containing an AscI site and an SfiI site. DNA products from amplifications 1 and 2 were joined using the "overlap" PCR method (amplification 3) (Horton, et al., Biotechniques, Vol. 8, pgs. 528-535 (1990)) with only the NotI primer and the SfiI primer. Complementarity between the AscI containing end of each initial DNA amplification product from reactions 1 and 2 allowed joining of these two pieces during amplification. Next the TPL was amplified (amplification 4) (sequences 6049 to 9730 of Ad-d1327 [identical to similar sequences from Ad5, Genbank accession #M73260]) from cDNA made from mRNA isolated from 293 cells (ATCC accession No. CRL 1573) infected for 16 hrs. with Ad-d1327 using primers containing SfiI and XbaI sites respectively. DNA fragments from amplification reactions 3 and 4 were then joined using PCR (amplification 5) with the NotI and XbaI primers, thus creating the complete gene block.

Third, the ITR-encapsidation signal-TPL fragment was then purified, cleaved with NotI and XbaI and inserted into the NotI, XbaI cleaved pHR plasmid. This plasmid was designated pAvS6A and the orientation was such that the NotI site of the fragment was next to the T7 RNA polymerase site (Figure 5).

Fourth, the SV40 early polyA signal was removed from SV40 DNA as an HpaI-BamHI fragment, treated with T4 DNA polymerase and inserted into the SalI site of the plasmid pAvS6A- (Figure 5) to create pAvS6 (Figures 5 and 6).

C. Production of Adenovirus Av1RE.

The apoB cDNA fragment was obtained from pCRE (Figure 2) after digestion with EcoRV and Ecl136 restriction enzymes. The apoB cDNA fragment was then ligated into the EcoRV-

linearized pAvS6 to generate pAvS6E (Figure 7), in which the apoB cDNA is placed downstream of the RSV promoter.

pAvS6E was linearized with KpnI restriction enzyme and co-transfected with the large ClaI fragment of Ad5d1327. This transfection produced the recombinant adenovirus containing the apoB cDNA and is referred to as Av1RE. A schematic diagram of Av1RE is shown in Figure 8. Recombinant adenoviral plaques were screened by PCR for the presence of apoB cDNA. Positive plaques were identified and were expanded in 293 cells. The adenovirus titers (particles/ml) were determined spectrophotometrically (Weiden, Proc. Nat. Acad. Sci., Vol. 91, pgs. 153-157 (1994); Halbert, J. Virol., Vol. 56, pgs. 250-257 (1985) and compared to the biological titer (pfu/ml). The ratio of total particles to infectious particles (particles/pfu) usually was 100 or less.

D. Construction of Av1LacZ4.

The recombinant, replication-deficient adenoviral vector Av1LacZ4, which expresses a nuclear-targetable B-galactosidase enzyme, was constructed in two steps. First, a transcriptional unit consisting of DNA encoding amino acids 1 through 4 of the SV40 T-antigen followed by DNA encoding amino acids 127 through 147 of the SV40 T-antigen (containing the nuclear targeting peptide Pro-Lys-Lys-Lys-Arg-Lys-Val), followed by DNA encoding amino acids 6 through 1021 of E. coli B-galactosidase, was constructed using routine cloning and PCR techniques and placed into the EcoRV site of pAvS6 to yield pAvS6-nlacZ (Figure 9).

The infectious, replication-deficient, Av1LacZ4 was assembled in 293 cells by homologous recombination. To accomplish this, plasmid pAvS6-nLacZ was linearized by cleavage with KpnI. Genomic adenoviral DNA was isolated from purified Ad-d1327 viruses by Hirt extraction, cleaved with ClaI, and the large (approximately 35 kb) fragment was isolated by agarose gel electrophoresis and purified. The ClaI fragment was used as the backbone for all first

generation adenoviral vectors, and the vectors derived from it are known as Av1.

Five micrograms of linearized plasmid DNA (pAvS6n-LacZ) and 2.5 μ g of the large ClaI fragment of Ad-d1327 then were mixed and co-transfected into a dish of 293 cells by the calcium phosphate precipitation method. After 16 hours, the cells were overlaid with a 1:1 mixture of 2% Sea Plaque agar and 2x medium and incubated in a humidified, 37°C, 5% CO₂/air environment until plaques appeared (approximately one to two weeks). Plaques were selected and intracellular vector was released into the medium by three cycles of freezing and thawing. The lysate was cleared of cellular debris by centrifugation. The plaque (in 300 μ l) was used for a first round of infection of 293 cells, vector release, and clarification as follows:

One 35 mm dish of 293 cell was infected with 100 μ l of plaque lysate plus 400 μ l of IMEM-2 (IMEM plus 2% FBS, 2mM glutamine (Bio Whittaker 046764)) plus 1.5 ml of IMEM-10 (Improved minimal essential medium (Eagle's) with 2x glutamine plus 10% vol./vol. fetal bovine serum plus 2mM supplemental glutamine (Bio Whittaker 08063A) and incubated at 37°C for approximately three days until the cytopathic effect, a rounded appearance and "grapeliike" clusters, was observed. Cells and supernatant were collected and designated as CVL-A. Av1LacZ4 vector (a schematic of the construction of which is shown in Figure 10) was released by three cycles of freezing and thawing of the CVL-A. Then, a 60 mm dish of 293 cells was infected with 0.5 ml of the CVL-A plus 3 ml of IMEM-10 and incubated for approximately three days as above. Cells and supernatant from this infection then were processed by three freeze/thaw cycles in the same manner. Av1LacZ4 also is described in Yei, et al., Human Gene Therapy, Vol. 5, pgs. 731-744 (1994); Trapnell, Advanced Drug Delivery Reviews, Vol. 12, pgs. 185-199 (1993), and Smith, et

al., Nature Genetics, Vol. 5, pgs. 397-402 (December 1993), which are incorporated herein by reference.

E. Transduction of HepG2 cells.

HepG2 cells were cultured in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum (FBS). Transductions of the cells with Av1RE or Av1LacZ4 were carried out in EMEM containing 2% FBS, 100 units/ml penicillin, and 10 μ g/ml streptomycin, and were carried out when the cells had reached approximately 90% confluency, and at multiplicities of infection (MOI) of 10 or 100. The adenoviral vector was diluted in 0.5 ml of the transduction medium, and was placed on the cell monolayer for 1.5 hours at 37°C. The medium was removed and 1 ml of fresh transduction medium then was added. After 24 hours, the medium was collected and Western analysis was carried out using a 10 μ l aliquot. The Western blot analysis was conducted using an anti-human apo E monoclonal antibody, 3H1. As shown in Figure 11, untransduced HepG2 cells and Av1LacZ4 transduced HepG2 cells secreted a low level of apo E which was detected in the culture medium (Figure 11, lanes 3 and 4). Av1RE transduced HepG2 cells, however, secreted substantially higher levels of apoE, indicating that the vector directs the overproduction of human apoE *in vitro*. (Figure 11, lanes 5 and 6.) Vector derived human apoE had an identical molecular weight of 34,000 daltons compared to both purified human apoE (Figure 11, lanes 1 and 2) and the endogenous apoE synthesized by HepG2 cells (Figure 11, lanes 3 and 4).

Example 2

Adenoviral Expression of Human ApoE *in vivo*

C57BL/6J apoE-deficient mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). These mice were homozygous for the disruption of the apoE gene (Piedrahita, et al., Proc. Nat. Acad. Sci., Vol. 89, pgs. 4471-4475 (1992); Zhang, et al., Science, Vol. 258, pgs. 468-471 (1992)). The mice were fed Purina mouse chow and allowed to

acclimate approximately 3 weeks prior to vector administration. Each mouse then was treated by tail vein injection with 5×10^{11} recombinant Av1RE or Av1LacZ4 adenovirus vector particles in 400 μ l Hank's buffered saline solution (HBSS).

Plasma cholesterol concentrations were determined before and after treatment using enzymatic methods (Sigma, St. Louis, Missouri). Blood was collected either from the retro-orbital plexus or the tail vein, and was transferred immediately to heparinized tubes. Plasma was collected following centrifugation at 7000 xg for 5 minutes. EDTA, Pefabloc, and apoprotinin were added to all plasma samples at final concentrations of 2 mM, 1 mM, and 10 μ g/ml, respectively.

A 1 μ l aliquot of plasma was denatured and applied to a 12% SDS PAGE gel (Novex). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using a mini transblot apparatus (Biorad, Hercules, CA) for 30 min. at 100 V. After the transfer was completed, the PVDF membrane was transiently stained with red Ponceau and the molecular weight standards were marked directly on the membrane. Molecular weight markers used ranged from 200 kDa to 14 kDa (Biorad). The PVDF membrane was blocked in 10 mM Tris, pH 7.4, containing 150 mM NaCl, 2 mM EDTA, 0.04% Tween 20 and 5% milk. The blocked membrane was first incubated for 1 hour at room temperature in a 1:3000 dilution of the primary antibody, anti-human apoE monoclonal, clone 3H1 (obtained from Dr. Y. Marcel, University of Ottawa). The membrane was developed with a secondary goat anti-mouse IgG1-horseradish peroxidase (HRPO) conjugated antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) using an enhanced chemiluminescence system (Amersham Lifesciences, Arlington Heights, IL). The membrane was exposed to film for approximately 1 to 10 sec. Purified human apoE (Calbiochem,

La Jolla, CA) was used as a positive control on all Western analysis.

Plasma was pooled from each treatment group and 200 μ l was applied to a Superose 6 gel filtration FPLC column (Pharmacia, Piscataway, NJ). The column was equilibrated in 10 mM Tris, pH 7.4 containing 150 mM NaCl, 2 mM EDTA, and 0.02% sodium azide at a flow rate of 0.35 ml/min and 0.5 ml fractions were collected. Cholesterol was determined on 100 μ l of each fraction by enzymatic methods. Purified human VLDL and HDL (Calbiochem) were used for calibration of the column.

At 7 days post injection, blood was obtained from the tail veins of the mice, and plasma was analyzed by Western blot analysis as described hereinabove. As shown in Figure 12, human apoE was detected in the plasma of the Av1RE-treated mice (lanes 8-12) and was not present in the plasma of the Av1LacZ4 vector control group (lanes 2-7).

Human apoE concentrations in mouse plasma also were quantitated by ELISA.

Human apoE in mouse plasma was measured using a sandwich type ELISA utilizing a mouse anti-human apoE monoclonal, 9-H8, as the capture antibody and a goat polyclonal as the detecting antibody. Values were determined using a standard curve obtained by including varying amounts of purified recombinant human apoE3 into plasma from apoE-deficient mice. Microtiter plates (Immulon 4, Dynatech) were coated with the 9-H8 antibody (5 μ g protein/ml) (Cappel, Durham, NC) in 100 mM sodium bicarbonate, pH9.6 overnight at room temperature. The monoclonal antibody solution was removed and the plate was washed 5 times using phosphate buffered saline (PBS) containing 0.05% Tween-20. Unless otherwise noted all subsequent incubations were carried out for one hour at room temperature. The nonspecific protein binding sites in each well were blocked using PBS containing 10% non-fat dry milk. The blocking buffer was removed and the plates were washed as

described previously. The standards and samples were diluted in PBS containing 2% bovine serum albumin (BSA) and allowed to incubate overnight. A 100 μ l aliquot of each sample or standard dilution was placed in individual wells and allowed to incubate. The plates were washed as described and were then incubated with 100 μ l of a 1:4000 dilution of the secondary anti-human apoE goat polyclonal antibody (Calbiochem, La Jolla, CA) in PBS containing 2% BSA. After washing, the plates were incubated with 100 μ l of a 1:3000 dilution of the tertiary, swine anti-goat IgG-HRPO polyclonal antibody (Caltag, San Francisco, CA). The reaction was developed using 100 μ l of 0.2 mg/ml 3,3',5,5',-tetramethylbenzidine (TMB), 0.01% H₂O₂, and was stopped by the addition of 100 μ l of 1M phosphoric acid. The absorbance at 450 nm was measured using 405nm as reference. The average concentrations in mouse plasma 7 days after vector administration was 1.2 μ g/ml, which is approximately 4% of normal human apoE levels.

The mean cholesterol value in apoE-deficient mice prior to vector treatment was 794 mg/dl (Zhang, 1992; Plump, Cell, Vol. 71, pgs. 343-353 (1992); Nakashima, et al., Arterioscler. Thromb., Vol. 14, pgs. 133-140 (1994); Ishibashi, et al., Proc. Nat. Acad. Sci., Vol. 91, pgs. 4431-4435 (1994)). As shown in Figure 13, one week after administration of the AvlRE vector, plasma cholesterol levels, indicated as average total plasma control concentrations (TPC), and determined as hereinabove described, had declined 8-fold to a mean value of 98 mg/dl, which is equivalent to levels found in normal C57BL6 mice fed a chow diet. (Lusis, et al., J. Biol. Chem., Vol. 262, pgs. 7594-7604 (1987); Zhang, 1992; Shimano, et al., Proc. Nat. Acad. Sci., Vol. 89, pgs. 1750-1754 (1992)). As indicated in Figure 13, the open bars denote the mice that were given AvlLacZ4, and the striped bars denote the mice treated with AvlRE. Mice that received the control AvlLacZ4 vector had

similar levels of plasma cholesterol before and after treatment, indicating that the reduction in plasma cholesterol concentrations was due to the expression of human apoE.

The plasma lipoprotein distribution of the Av1RE and Av1LacZ4 treated mice 7 days after vector administration is shown in Figure 14. Pooled plasma from each treatment group was fractionated using a Superose 6 gel filtration column as hereinabove described, and cholesterol content was measured in each fraction across the elution profile. As shown in Figure 14, the open circles denote plasma from the Av1LacZ4 treated group, and the dark circles denote plasma from the mice treated with Av1RE. As expected for untreated apoE-deficient mice, the lipoprotein elution profile of the Av1LacZ4-treated group showed that the majority of the cholesterol eluted in the VLDL/LDL region. (Zhang, et al., 1992; Plump, et al., 1992.) In contrast, the lipoprotein distribution of the Av1RE-treated animals was shifted such that the cholesterol found in the VLDL/LDL region was reduced and that HDL was the primary cholesterol-containing lipoprotein.

The plasma lipoprotein distribution of human apoE in the Av1RE-treated mice was confirmed by Western analysis of the fractionated plasma samples (Figure 15). The FPLC fraction numbers from Figure 14 are listed above each lane. The majority of the human apoE was associated with the VLDL/LDL fraction, although a smaller proportion of the apoE was detected in the HDL fraction.

Figures 16A and 16B show total plasma cholesterol and plasma human apoE concentrations after adenoviral vector administration. Open circles denote the Av1LacZ4-treated mice, and black circles denote the Av1RE-treated mice. Plasma human apoE concentrations were determined by ELISA as hereinabove described. As shown in Figure 16A, the reduction in total plasma cholesterol levels in the Av1RE-treated apoE-

deficient mice persisted for at least 21 days after administration of the adenoviral vector. The mean plasma cholesterol concentrations in the Av1RE-treated apoE-deficient mice were approximately 150 mg/dl over the first 21 days although a greater variation was found between individuals at 14 and 21 days. At 35 days after vector administration, the plasma cholesterol concentrations in the Av1RE-treated group increased to approximately 550 mg/dl, but this was lower than that seen in the control vector group. No significant change in the plasma cholesterol concentrations was observed over the course of the study in Av1LacZ4-treated control mice.

Expression of human apoE in mouse plasma persisted for at least 35 days after administration of the adenoviral vector Av1RE, as shown in Figure 16B. The concentration of human apoE varied over the course of the study, with the highest level of 3.4 μ g/ml found at 14 days. The increase in plasma cholesterol concentration at 35 days post injection correlated with a decline in the plasma human apoE concentrations to an average level of 0.5 μ g/ml (Figure 16B).

The results in this example showed that adenovirus mediated expression of human apoE in hypercholesterolemic apoE-deficient mice resulted in a transient phenotypic reversion of the hypercholesterolemic state normally found in this mouse strain. The levels of human apoE expression were sufficient to produce a significant lowering of plasma cholesterol concentrations for at least 21 days after vector administration. Thus, correction of hypercholesterolemic conditions by direct *in vivo* gene transfer may be employed for the treatment of hyperlipidemias due to apoE deficiency (Schaefer, et al., J. Clin. Invest., Vol. 78, pgs. 1206-1219 (1986)) or dysfunction (Rall, et al., J. Internal Med., Vol. 231, pgs. 653-659 (1992)).

An inverse relationship between plasma apoE concentrations and plasma cholesterol levels has been

reported previously in transgenic mice expressing the rat and human apoE genes. (Shimano, et al., Proc. Nat. Acad. Sci., Vol. 89, pgs. 1750-1754 (1992); Smith, et al., J. Biol. Chem., Vol. 265, pgs. 14709-14712 (1990)). The above results showed that a human apoE concentration of 1 to 4 $\mu\text{g/ml}$ was sufficient to lower plasma cholesterol levels to the normal range in apoE-deficient mice. Thus, severe hypercholesterolemia due to gene knockout is affected by relatively modest apoE levels. In addition to the treatment of hyperlipidemias, the AvlRE vector also could be used to assess the effects of human apoE gene therapy on the development of Alzheimer's disease.

The decrease in plasma cholesterol levels observed in the AvlRE-treated mice was accompanied by changes in the plasma lipoprotein distribution (Figure 14). The presence of human apoE in the plasma of the apoE-deficient mice produced a decrease in VLDL/LDL cholesterol and an increase in HDL cholesterol. ApoE-deficient mice have both intestinal- and liver-derived remnant lipoprotein particles that accumulate in plasma and result in elevated plasma cholesterol concentrations (Zhang, et al., 1992; Plump, et al., 1992). These remnant particles are normally cleared from the circulation via the interaction of apoE with either the LDLR and/or the LRP (Ishibashi, et al., Proc. Nat. Acad. Sci., Vol. 91, pgs. 4431-4435 (1994); Willnow, et al., Science, Vol. 264, pgs. 1471-1474 (1994)). Previous studies have shown that the elevation of apoE levels can enhance the clearance of VLDL, LDL, and chylomicrons from the circulation (Shimano, et al., J. Clin. Invest., Vol. 90, pgs. 2084-2091 (1992); Shimano, et al., J. Clin. Invest., Vol. 93, pgs. 2215-2223 (1994)). The reduction of plasma cholesterol concentrations and changes in the plasma lipoprotein distribution was presumably a result of the association of the human apoE protein with both apoB48- and apoB100-remnant lipoprotein particles (Figure 15), thereby increasing their

removal from the circulation. The current study demonstrates that the phenotypic correction of the apoB-deficient mouse can be achieved by the transient delivery of the apoE3 gene using an adenoviral vector.

The disclosure of all patents, publications, including published patent applications, and database entries referenced in this specification are specifically incorporated herein by reference in their entirety to the same extent as if each such individual patent, publication, and database entry were specifically and individually indicated to be incorporated by reference.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: McClelland, Alan
Stevenson, Susan C.
Teng, Ba Bie

(ii) TITLE OF INVENTION: GENE THERAPY FOR
HYPERCHOLESTEROLEMIA

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:

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(C) CITY: Roseland
(D) STATE: New Jersey
(E) COUNTRY: USA
(F) ZIP: 07068

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5 inch diskette
(B) COMPUTER: IBM PS/2
(C) OPERATING SYSTEM: MS-DOS
(D) SOFTWARE: WordPerfect 5.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Olstein, Elliot M.
- (B) REGISTRATION NUMBER: 24,025
- (C) REFERENCE/DOCKET NUMBER: 271010-228

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 201-994-1700
- (B) TELEFAX: 201-994-1744

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURE:

- (A) NAME/KEY: PCR primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ACTCAGCCCC AGCGGAGGTG AAGGACGTCC TTCCCCAGGA GCCG

44

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURE:

(A) NAME/KEY: PCR primer

(ix) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TTCCTCCAGT TCCGATTGT AGGCCTCAA CTCCTCATG GTCTCGTC

48

(2) INFORMATION SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURE:

(A) NAME/KEY: PCR primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCCTACAAAT CGGAACTGGA GGAA

24

(2) INFORMATION FOR SEQ ID NO: 4:

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- (A) LENGTH: 23 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURE:

(A) NAME/KEY: PCR primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGGCTTCGGC GTTCAGTGAT TGT

23

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Pro Lys Lys Lys Arg Lys Val

5

WHAT IS CLAIMED IS:

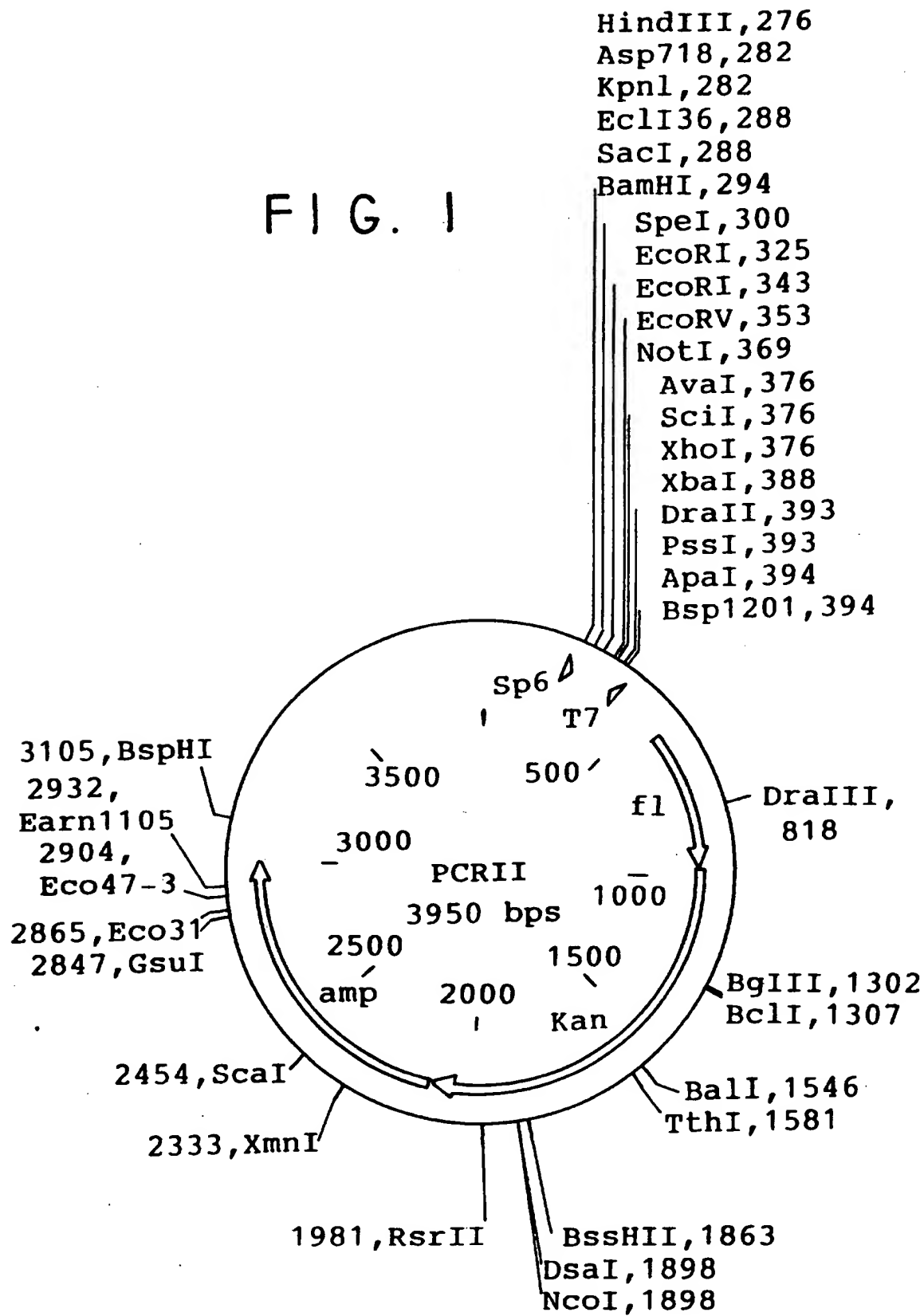
1. An adenoviral vector including a nucleic acid sequence encoding apolipoprotein E or a fragment or derivative thereof having the biological activity of apolipoprotein E.
2. The vector of Claim 1 wherein said adenoviral vector is modified such that at least a portion of the adenoviral genome has been deleted.
3. The vector of Claim 2 wherein said vector comprises an adenoviral 5' ITR; an adenoviral 3' ITR; an adenoviral encapsidation signal; a DNA sequence encoding apolipoprotein E or a fragment or derivative thereof; and a promoter controlling said DNA sequence encoding apolipoprotein E or a fragment or derivative thereof, wherein said vector is free of at least the majority of adenoviral E1 and E3 DNA sequences.
4. The vector of Claim 3 wherein said vector is Av1RE as shown in Figure 8.
5. A method of treating hypercholesterolemia in a host, comprising:

administering to a host an adenoviral vector including a nucleic acid sequence encoding apolipoprotein E or a fragment or derivative thereof, said adenovirus being administered in an amount effective to treat hypercholesterolemia in a host.
6. The method of Claim 5 wherein said adenoviral vector is administered in an amount of from 1 pfu to about 10^{14} pfu.
7. The method of Claim 6 wherein said adenoviral vector is administered in an amount of from 10^6 pfu to about 10^{13} pfu.
8. Eukaryotic cells transduced with the adenoviral vector of Claim 1.
9. A method of treating hypercholesterolemia in a host, comprising:

administering to a host the eukaryotic cells of Claim 8 in an amount effective to treat hypercholesterolemia in a host.

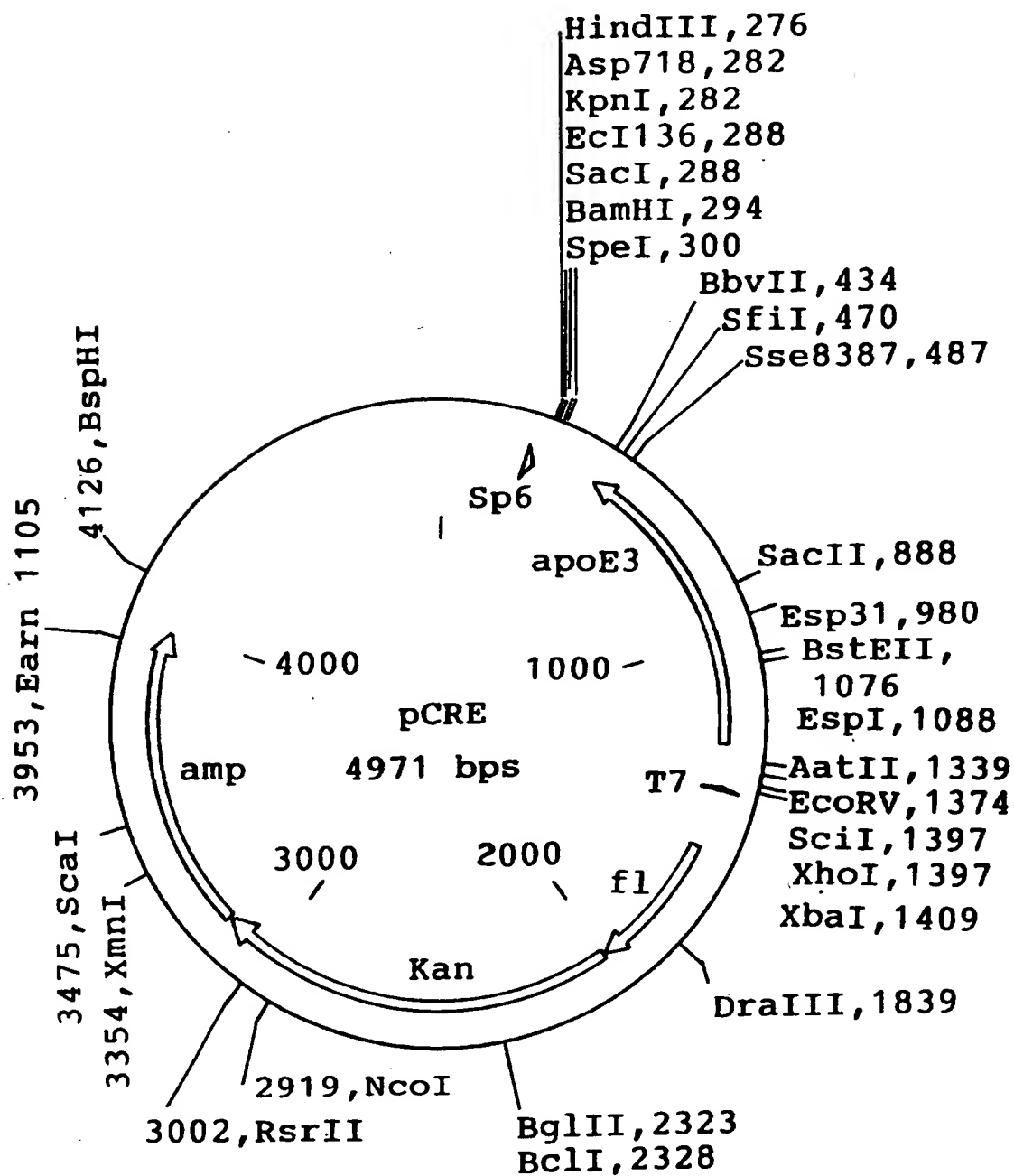
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FIG. 1



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FIG. 2



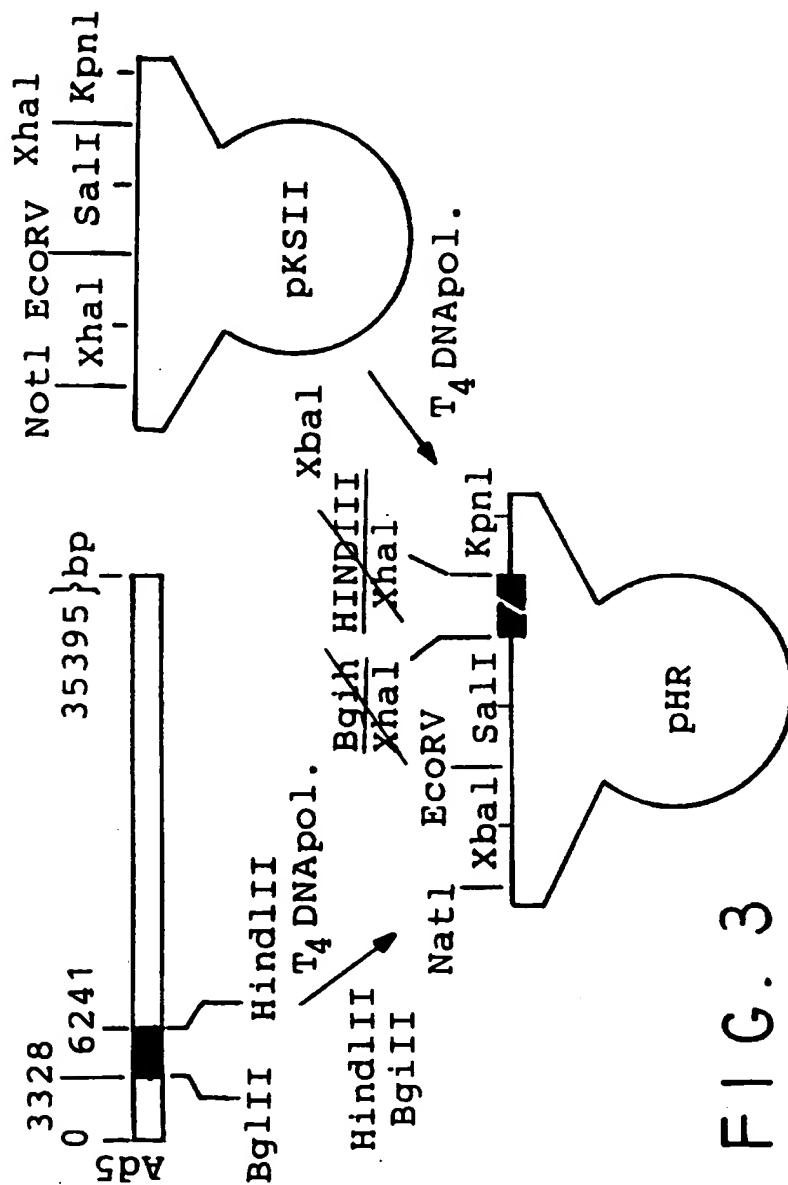
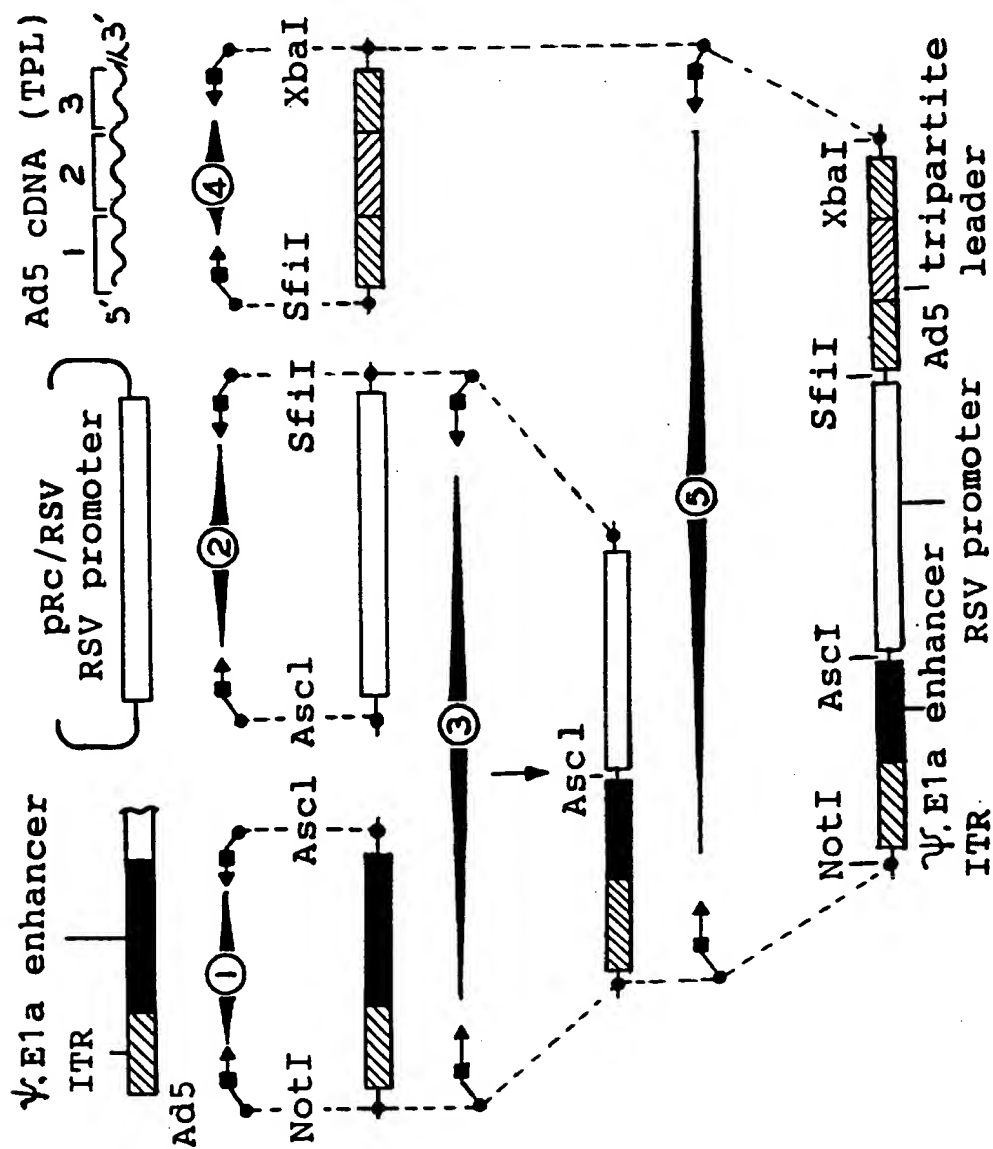


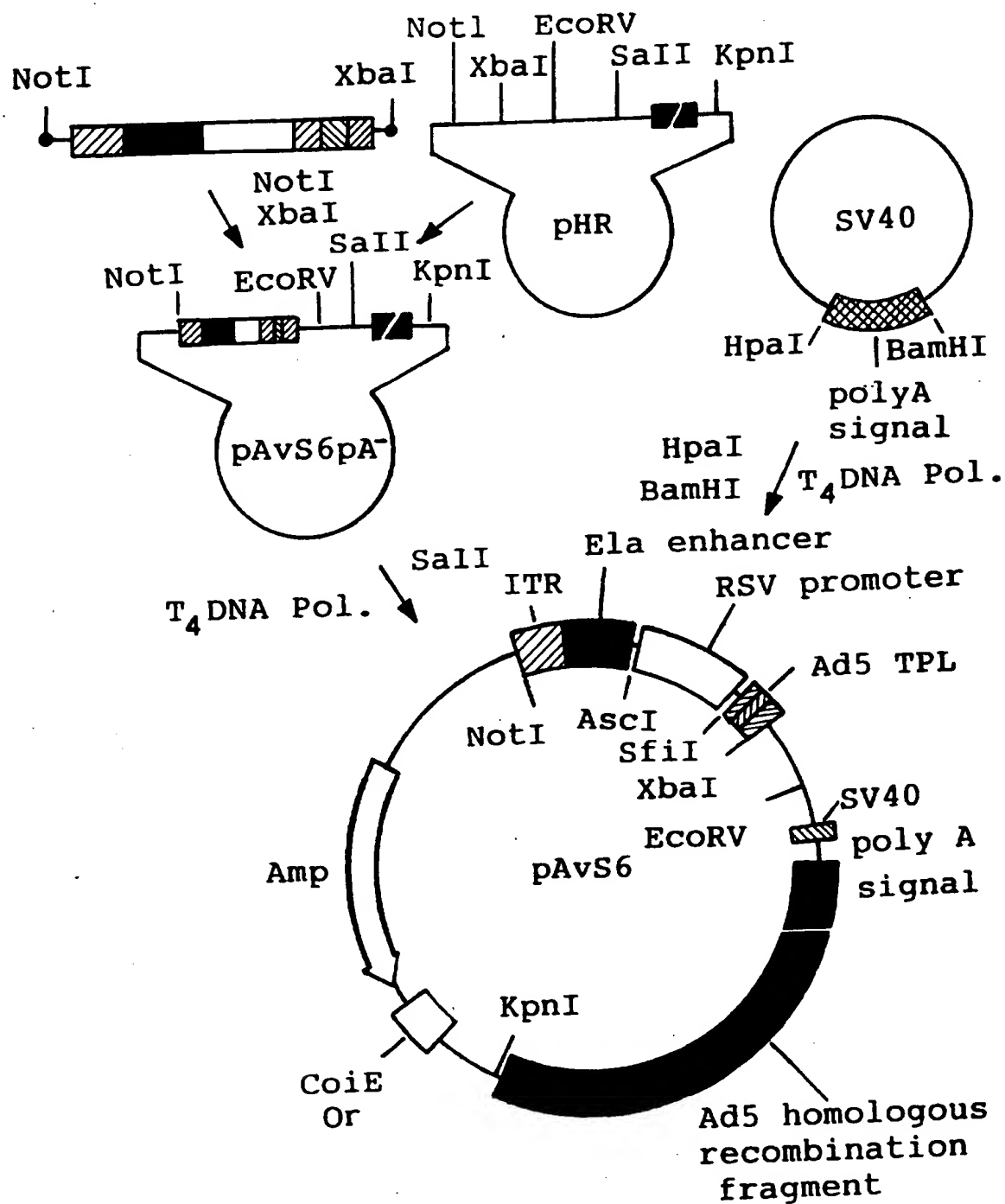
FIG. 3

FIG. 4



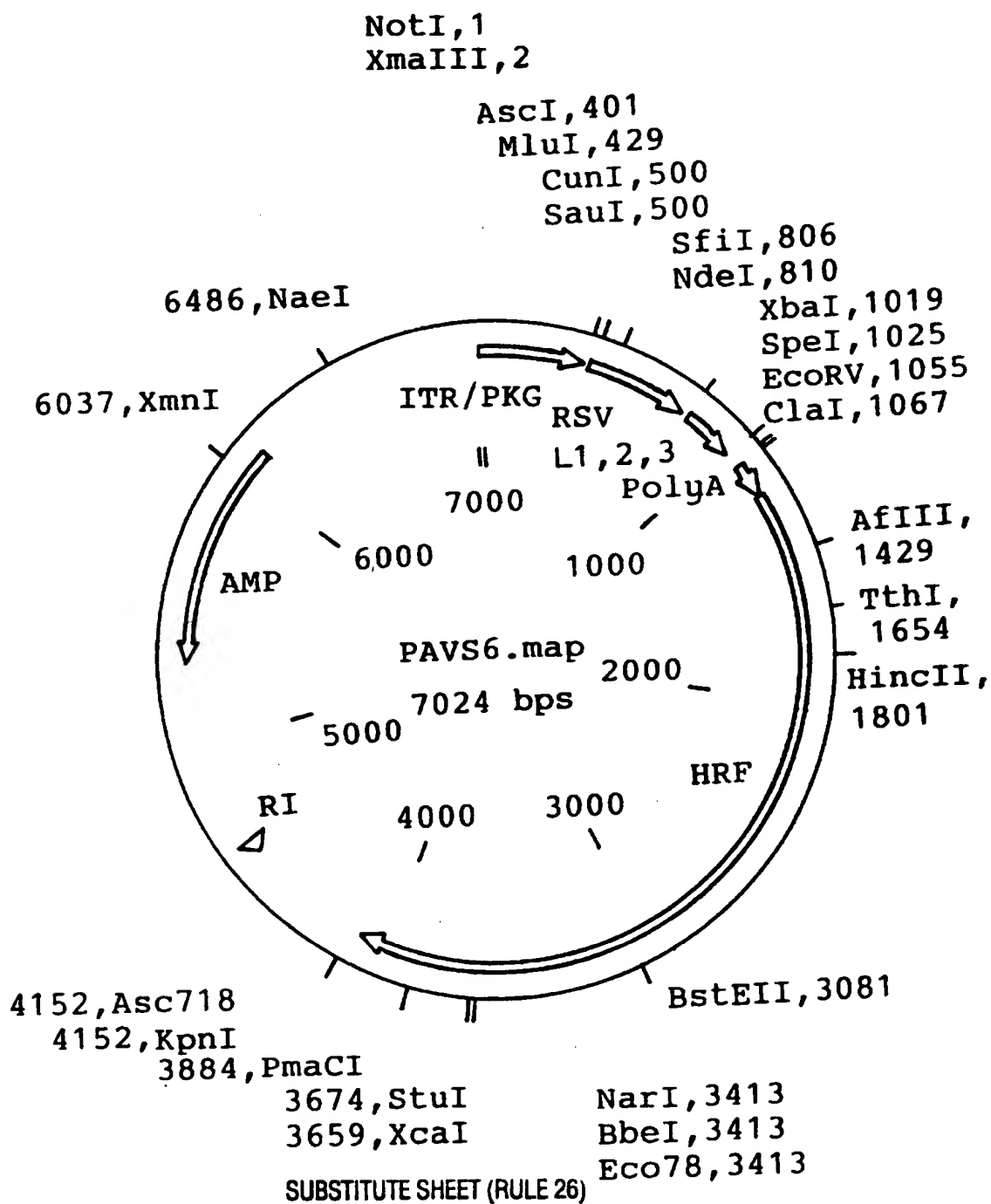
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FIG. 5



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FIG. 6



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FIG. 7

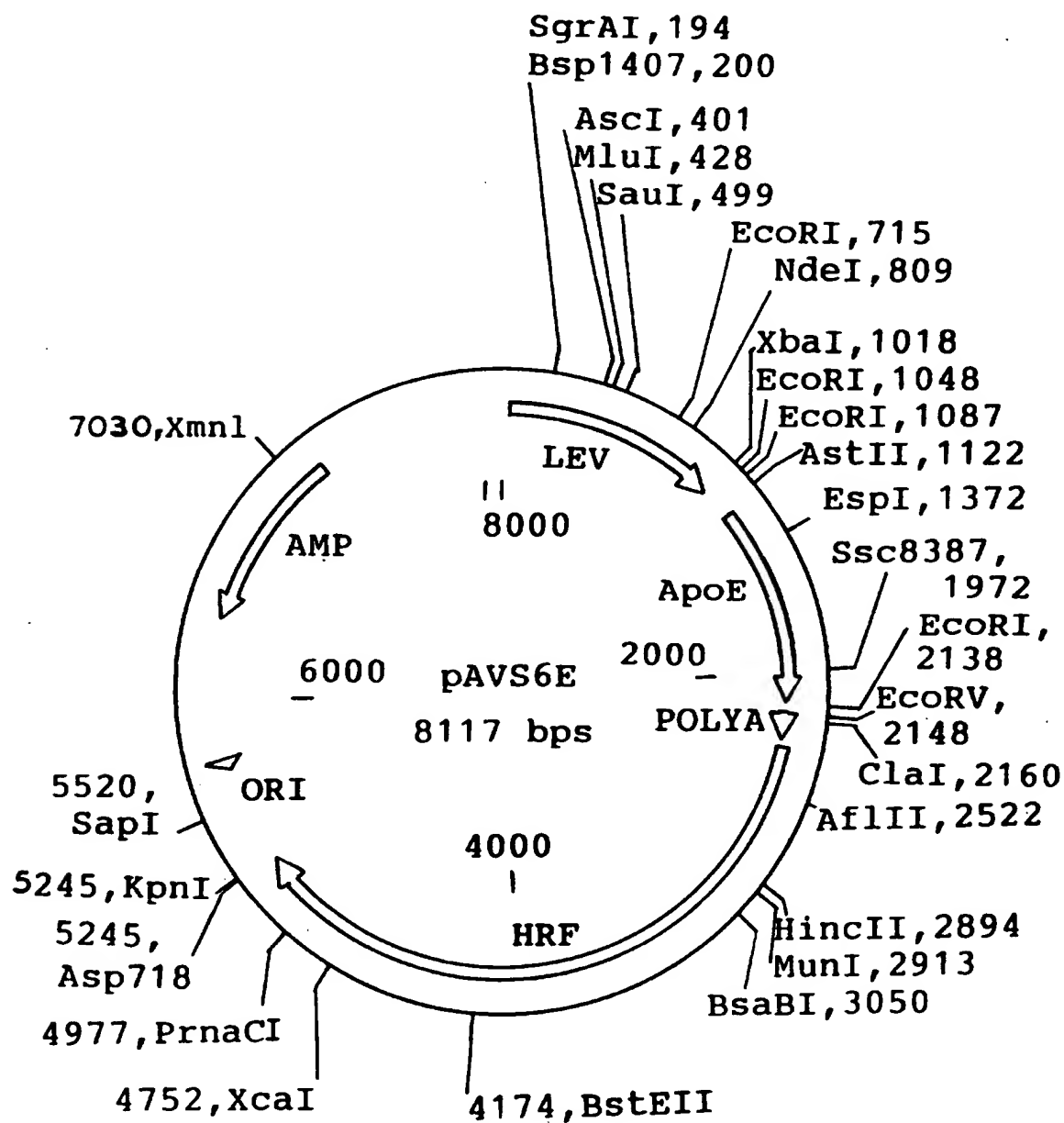
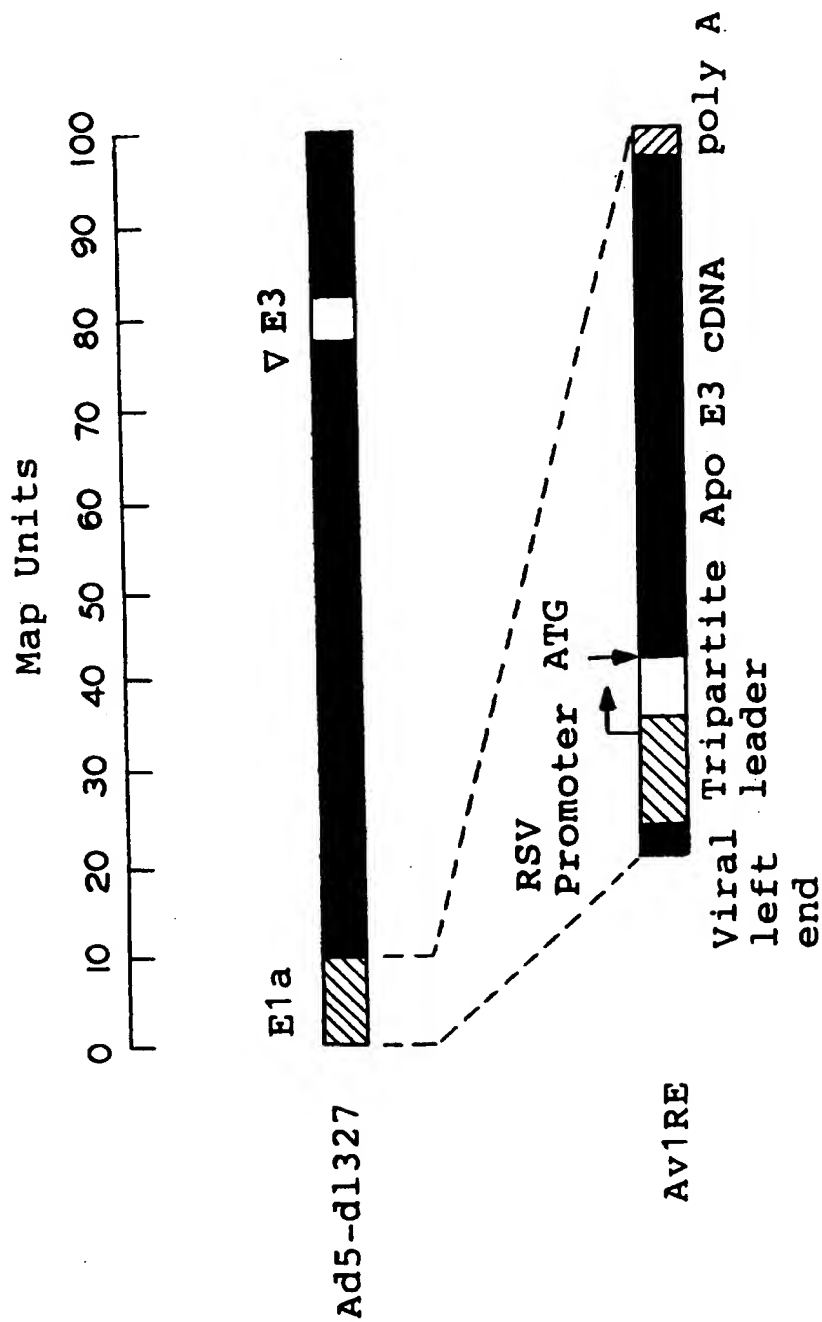
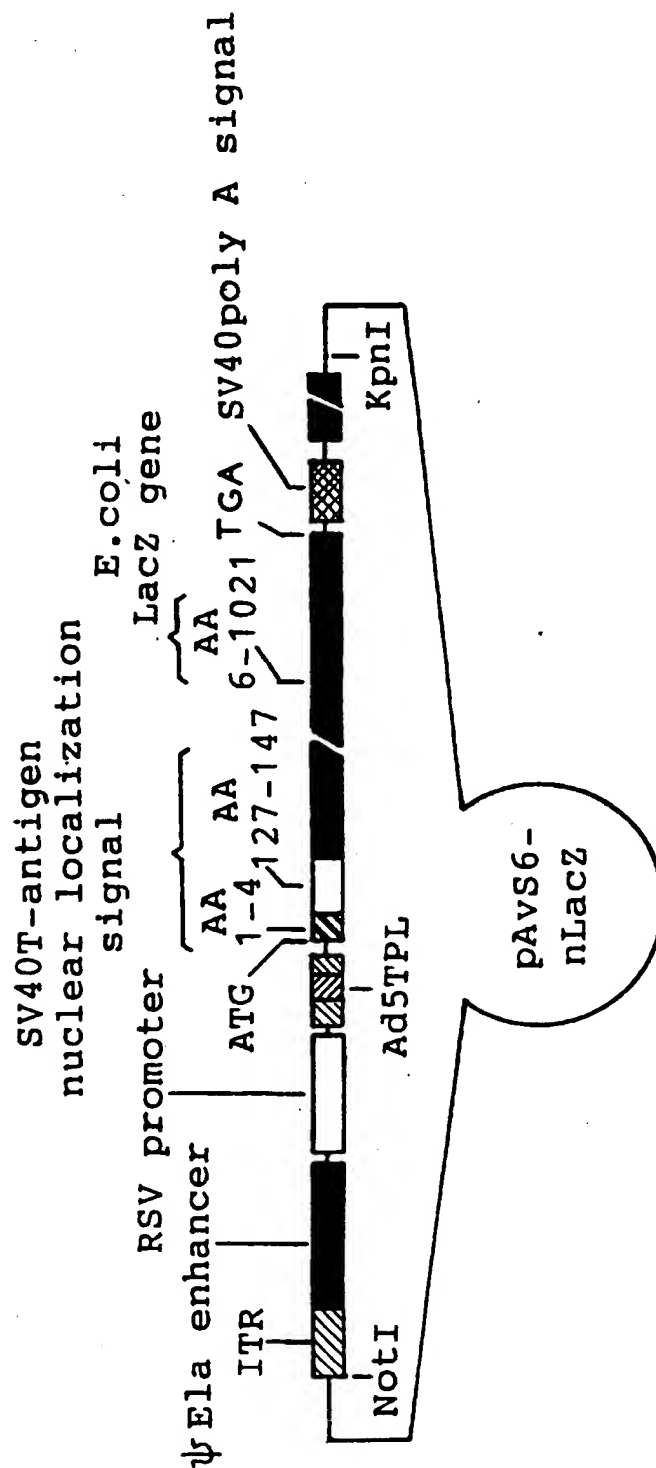


FIG. 8



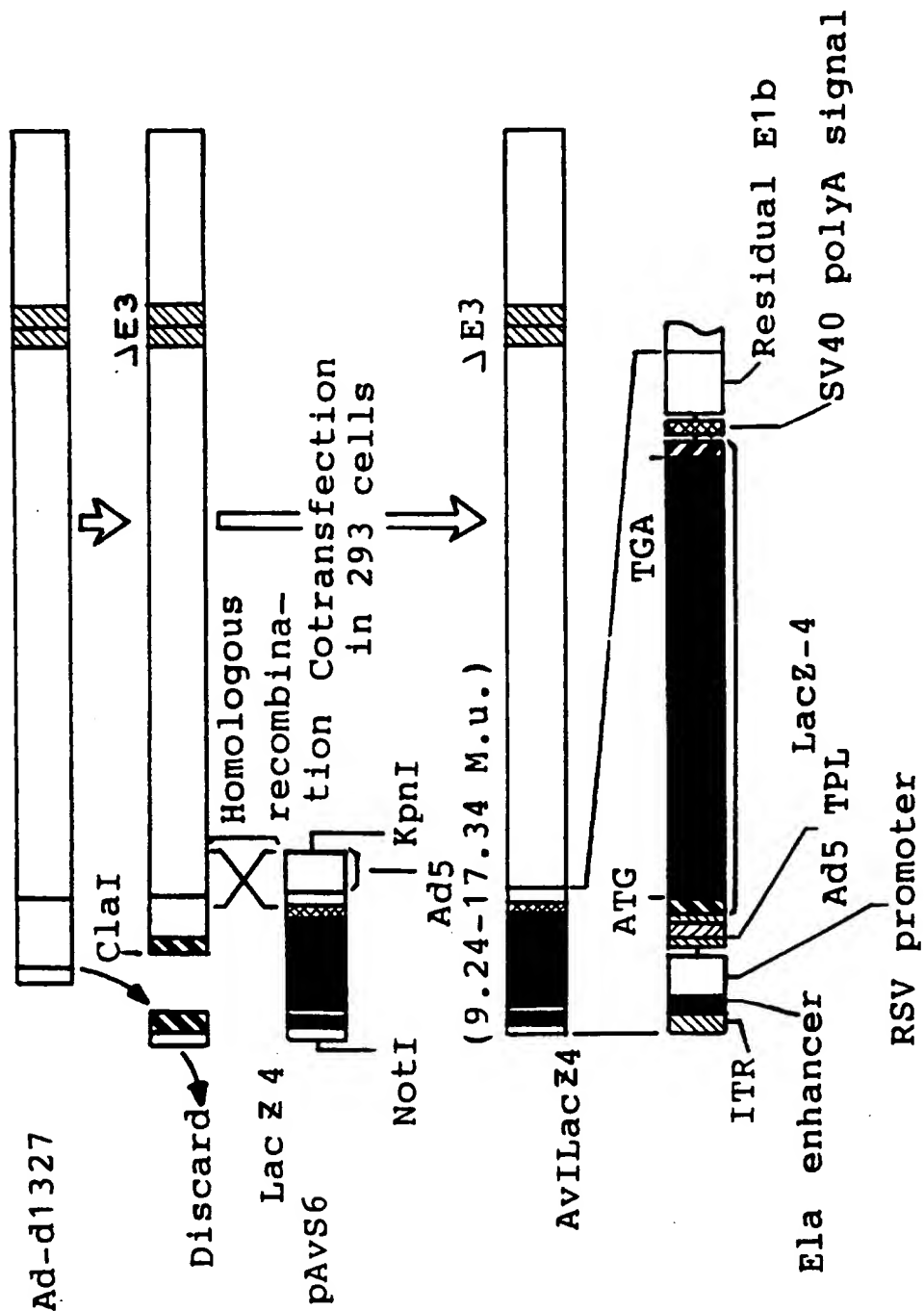
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FIG. 9



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FIG. 10



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FIG. 11

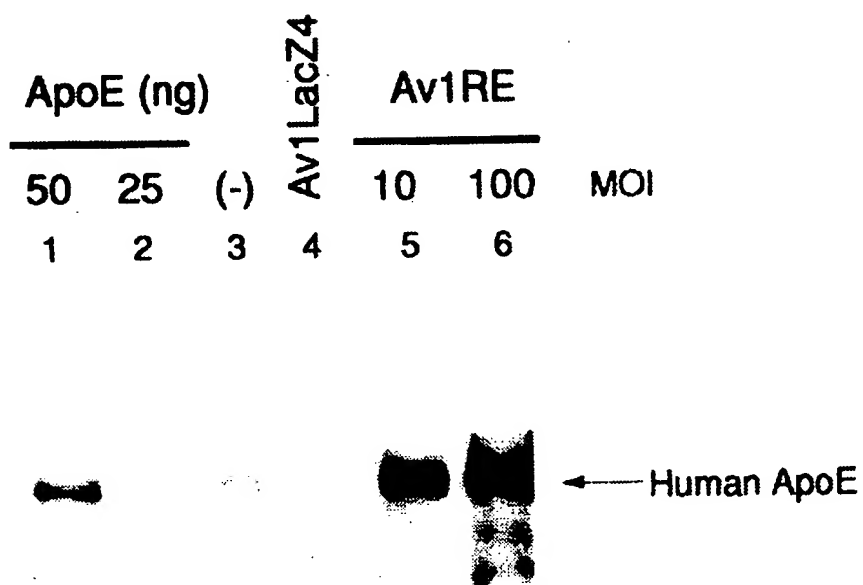
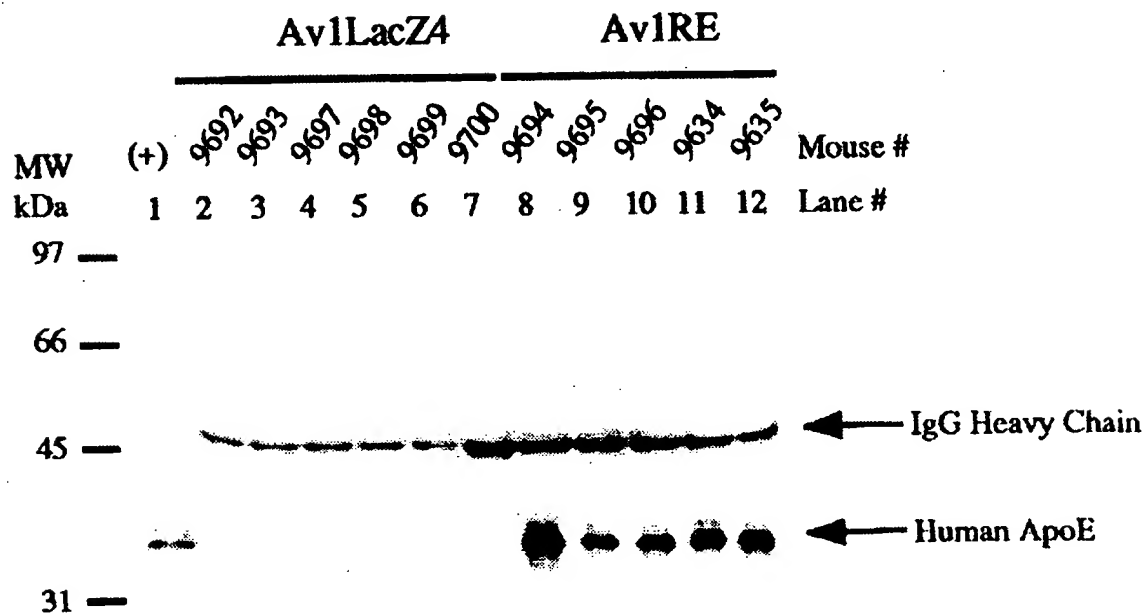
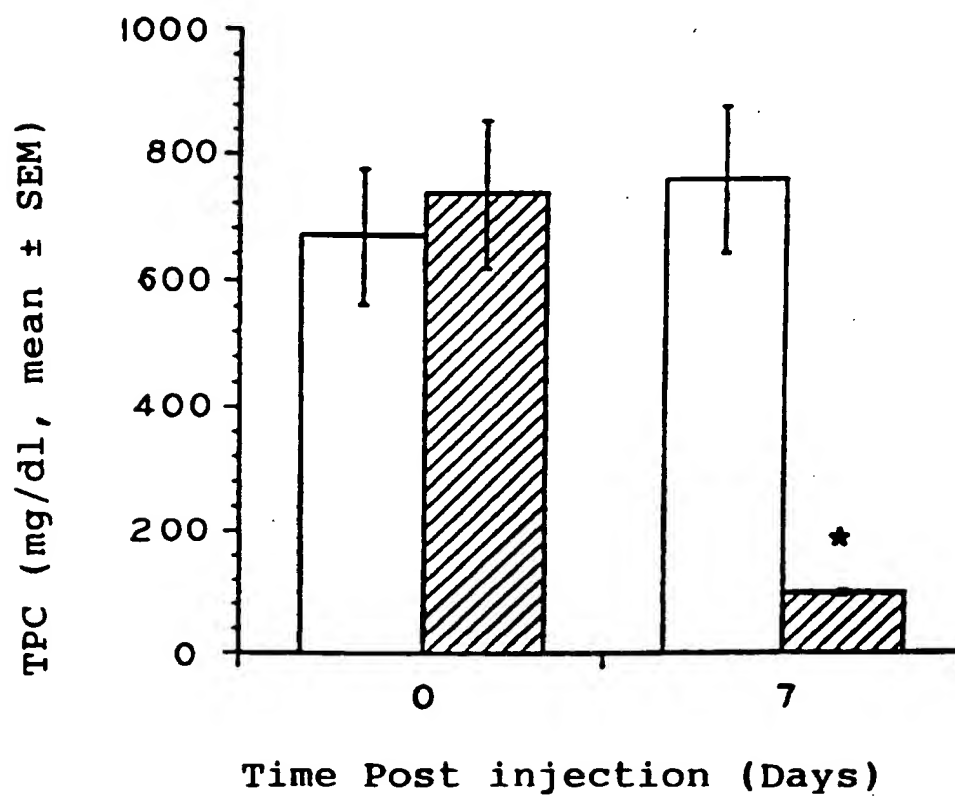


FIG. 12



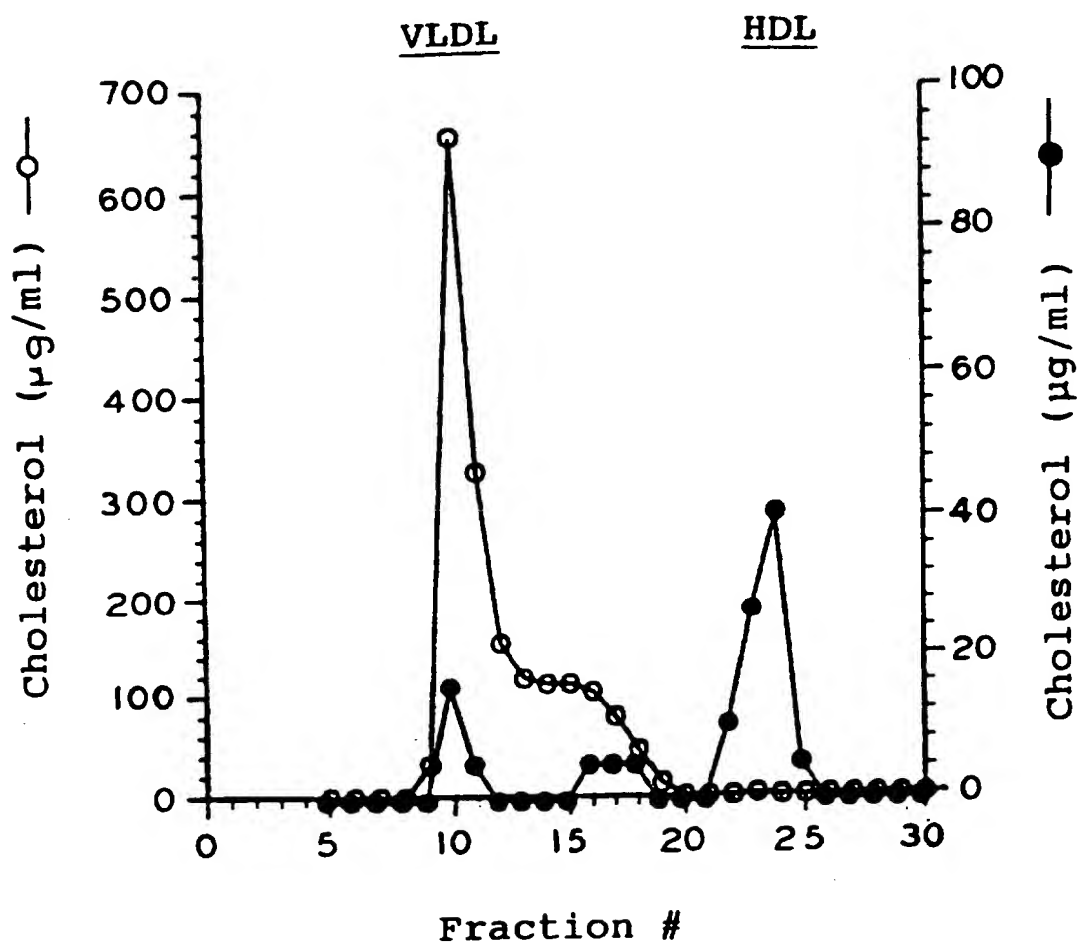
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FIG. 13



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FIG. 14



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FIG. 15

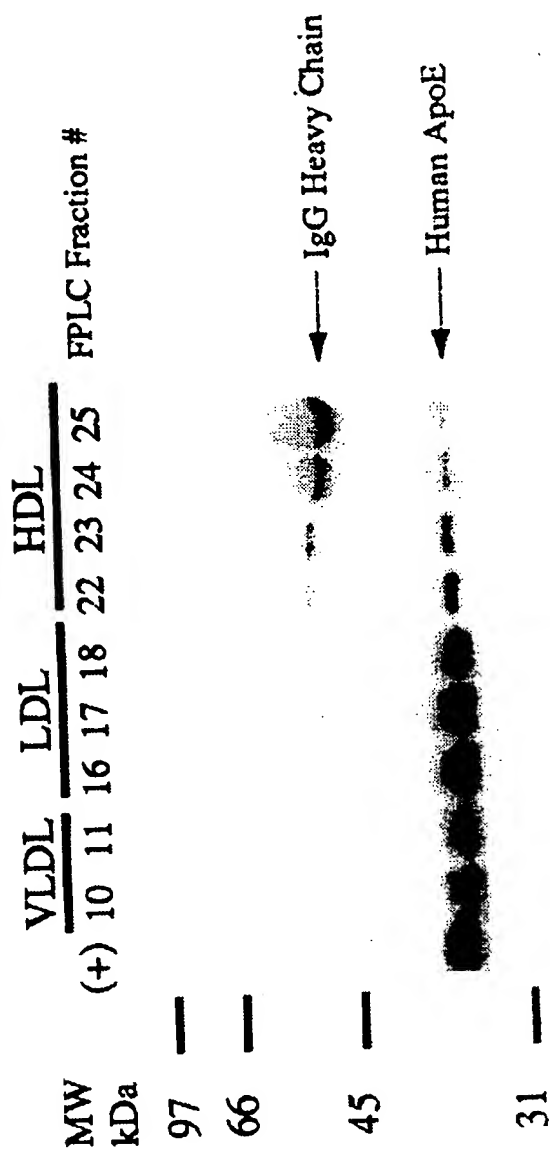


FIG. 16 A

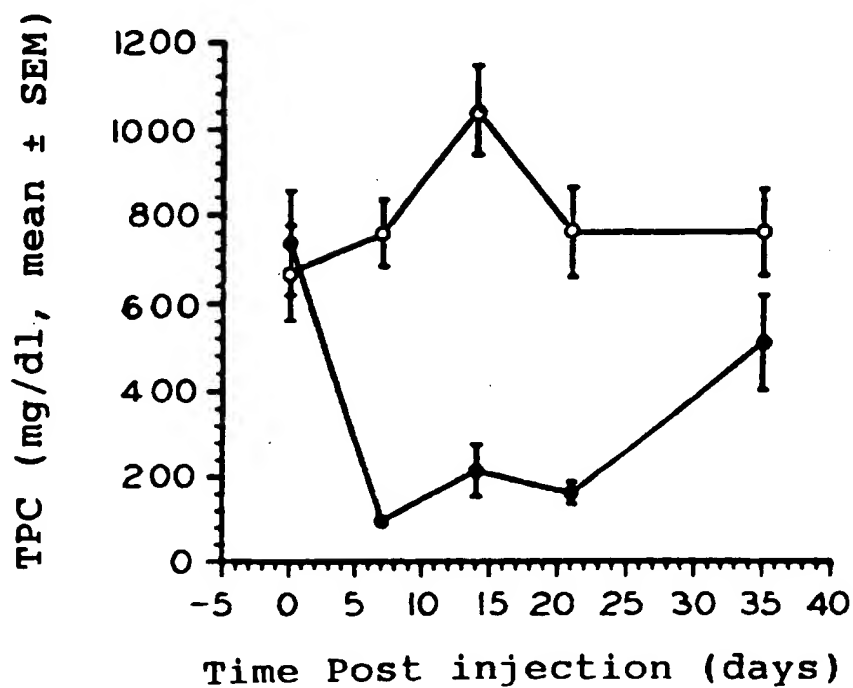
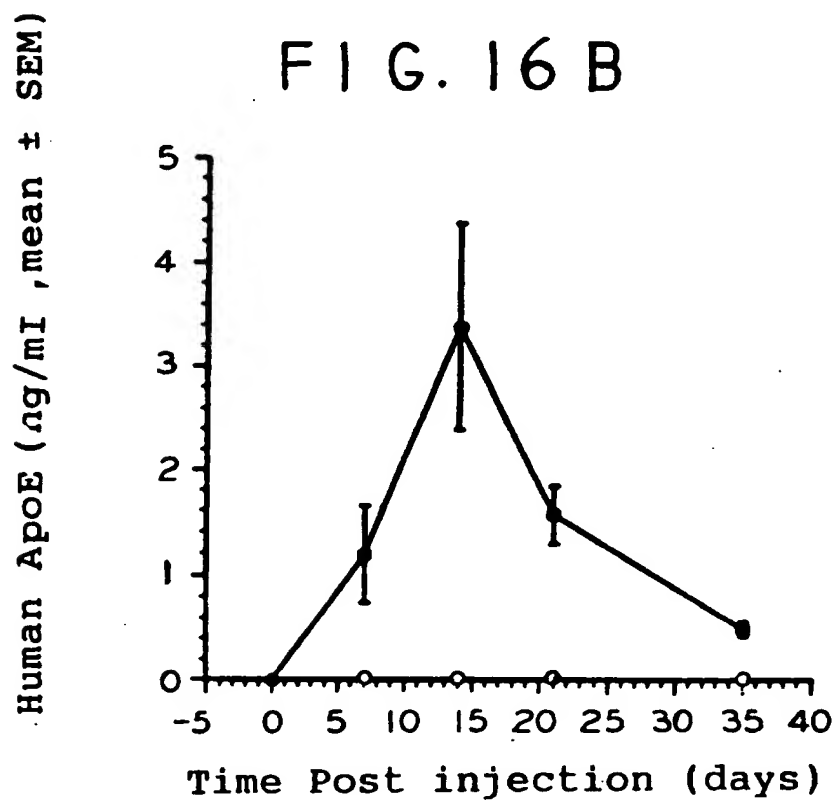


FIG. 16 B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14541

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 31/00

US CL : 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	THE JOURNAL OF CLINICAL INVESTIGATION, Vol. 96, issued September 1995, Kashyap et al, "Apolipoprotein E Deficiency in Mice: Gene Replacement and Prevention of Atherosclerosis Using Adenovirus Vectors", pages 1612-1620, see entire document.	1-9
A,P	CIRCULATION, Vol. 91, No. 2, issued 15 January 1995, Dammerman et al, "Genetic Basis of Lipoprotein Disorders", pages 505-512, see entire document.	5-9
A	JAMA, Vol. 256, No. 1, issued 02 January 1991, Mahley et al, "Genetic Defects in Lipoprotein Metabolism", pages 78-83, see entire document.	5-9

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

X	FURTHER DOCUMENTS ARE ALSO OF INTEREST			
•	Special categories of cited documents:	T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
A	document defining the general state of the art which is not considered to be of particular relevance	X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	
E	earlier document published on or after the international filing date	Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	
L	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	A*	document member of the same patent family	
O	document referring to an oral disclosure, use, exhibition or other means			
P	document published prior to the international filing date but later than the priority date claimed			

Date of the actual completion of the international search
02 JANUARY 1996

Date of mailing of the international search report
08 FEB 1996

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Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14541

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ANNALS NEW YORK ACADEMY OF SCIENCES, Vol. 598, issued 1990, Weisgraber et al, "Atherogenic Lipoproteins Resulting from Genetic Defects of Apolipoproteins B and E", pages 37-48, see entire document.	5-9
Y	Proceedings of the National Academy of Sciences, Vol. 90, issued April 1993, Herz et al, "Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice", pages 2812-2816, see entire document.	1-9
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 269, No. 18, issued 06 May 1994, Kozarsky et al, "In Vivo Correction of Low Density Lipoprotein Receptor Deficiency in the Watanabe Heritable Hyperlipidemic Rabbit with Recombinant Adenovirus", pages 13695-13702, see entire document.	1-9
Y	SOMATIC CELL AND MOLECULAR GENETICS, Vol. 19, No. 5, issued 1993, Kozarsky et al, "Adenovirus-Mediated Correction of the Genetic Defect in Hepatocytes from Patients with Familial Hypercholesterolemia", pages 449-458, see entire document.	1-9
Y	NATURE GENETICS, Vol. 5, issued December 1993, Smith et al, "Adenovirus mediated expression of therapeutic plasma levels of human factor IX in mice", pages 397-402, see entire document.	1-9
Y	JOURNAL OF CELLULAR BIOCHEMISTRY, Vol. 18, issued 1994, Kaleko et al, "ADENOVIRAL VECTOR-MEDIATED EXPRESSION OF THERAPEUTIC LEVELS OF HUMAN FACTOR IX IN MICE", page 242, abstract no. DZ 405, see entire document.	1-9
Y	JOURNAL OF CELLULAR BIOCHEMISTRY, Vol. 18, issued 1994, Kozarsky et al, "IN VIVO CORRECTION OF LDL RECEPTOR DEFICIENCY IN THE WATANABE HERITABLE HYPERLIPIDEMIC RABBIT WITH RECOMBINANT ADENOVIRUSES", page 242, abstract no. DZ 406, see entire document.	1-9
Y	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Vol. 200, No. 1, issued 15 April 1994, Fazio et al, "ACCUMULATION OF HUMAN APOLIPOPROTEIN-E IN RAT PLASMA AFTER IN VIVO INTRAMUSCULAR INJECTION OF NAKED DNA", pages 298-305, see entire document.	1-9

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US95/14541**C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 94/10322 (HERZ ET AL) 11 MAY 1994, see entire document.	1-9